TITLE: CONTINUOUS FREE CORTISOL PROFILES – CIRCADIAN RHYTHMS IN HEALTHY MEN

AUTHORS: R Bhake[1,2], V Kluckner[3], H Stassen[3], GM Russell[2], J Leendertz[2], K Stevens[4], ACE Linthorst[5*], SL Lightman[2,6*]

Affiliations:

[1]University Hospitals Leicester NHS Trust, Leicester LE1 5WW (Work carried out[2]);

[2]Henry Wellcome Laboratories for Integrative Neuroscience and Endocrinology;

[3]Department of Psychiatry, Psychotherapy & Psychosomatics, University of Zurich;

[4]Medical Statistics, Faculty of Health: Medicine, Dentistry and Human Sciences, University of Plymouth;

[5]Bristol Medical School, University of Bristol, BS1 3NY;

[6]University Hospitals Bristol NHS Foundation Trust, Bristol BS2 8HW.

* Shared Senior Authorship

ABSTRACT:

Context: The pituitary-adrenal axis had historically been considered a representative model for circadian rhythms. A recently developed portable collection device provided the opportunity to evaluate free cortisol profiles using the microdialysis approach in individuals free to conduct their day-to-day activities in their own surroundings.

Methods: Two separate experiments were conducted in healthy male volunteers – ten-minutely total and subcutaneous free cortisol were measured for 24-hour period in one and twenty-minutely subcutaneous free cortisol for 72 consecutive hours in free-living individuals in the other experiment.

Results: The characteristic circadian rhythm was evident in both serum total and subcutaneous free cortisol with the lowest levels being achieved and maintained in the hours surrounding sleep onset with peak levels occurring in every individual around waking. In all free-living individuals, the circadian rhythm was consistent across 72-hours despite a wide range of activities. All participants also showed increased cortisol following the consumption of lunch. The lowest levels during all 24 hour periods were observed during the hours following lights switch-off, at the onset of sleep

Conclusions: This is the first study to show up to three consecutive 24-hour measurements of subcutaneous free cortisol in healthy individuals. This, we believe is a landmark study that paves the way for ambulatory monitoring of free cortisol profiles continuously up to a period of 72 hours in a free-living individual going about their day to day activities whether in health or in diseases involving the HPA axis.

INTRODUCTION:

The hypothalamic-pituitary-adrenal (HPA) axis coordinates the secretion of glucocorticoids, principally cortisol in man and corticosterone in rodents, optimal concentration of which is integral to the maintenance of normal physiological functions and the recovery from stressful situations. Although corticosteroids secreted by the adrenal gland are not bound to protein[1]once they reach the circulation 95% of the hormone is rapidly bound by either corticosteroid-binding globulin (CBG)[1], or by albumin[2] which bind cortisol in equimolar ratios of 1:1 or a molar ratio of up to 1:10 respectively. It is only the small fraction of free unbound cortisol[1] that is able to pass out of the circulation and bind glucocorticoid receptors in the cells within glucocorticoid responsive target organs.

Microdialysis, as a well-established technique for measurement of molecules in extracellular fluid offers three significant advantages. First, it enables the measurement of the active free component of cortisol that is not protein-bound. It is a minimally invasive technique that does not involve venous cannulation or the removal of blood and is relatively free of risk. Repeated blood sampling, the mainstay of human (HPA axis) hormone testing for decades, in contrast, although routine is best carried out at a clinical facility for safety. As a result, little is known about dynamic functioning of hormonal systems in the most prevalent, and perhaps more relevant, physiological setting of an individual's own environment. In contrast, by virtue of its safety and lack of need for venous access, microdialysis is feasible in the home/work setting and together with a miniaturised collecting system is suitable for ambulatory sampling.

Free cortisol has been measured by microdialysis in healthy controls and cohorts of patients admitted to hospital with various medical conditions including those undergoing elective coronary artery bypass graft[3], critically ill patients[4] with septic shock[5,6], and with burns[7]. Equilibrium dialysis[3,4] or ultrafiltration[5] methods were used in blood samples, and microdialysis in the subcutaneous adipose tissue[6], dermis[7] and brain[8] in others. However, none of these studies were dynamic, looking at multiple samples over a short period of time to examine changes over time.

The term circadian rhythm applies to a periodicity of approximately 24 hours i.e. a day[9], and rhythms of duration shorter than 24 hours are ascribed the term ultradian[10]. All mammals that have been investigated to date exhibit both circadian and ultradian rhythms, which also operate at the level of other hormonal axes e.g. insulin[11], but the pituitary-adrenal axis had been historically considered a representative model for these rhythms[12] and a distinct circadian pattern is consistently found across members of an individual species[13]. The characteristic circadian and ultradian rhythm of free corticosterone has been demonstrated in the rat[14,15] there has been no equivalent study in man.

The use of our recently developed portable collection device[16], provided us with the opportunity for the prolonged evaluation of free cortisol profiles in individuals going about their day-to-day activities in their own surroundings. This initial study in normal subjects should also open up the feasibility of investigating conditions associated with abnormalities of HPA function.

MATERIALS AND METHODS:

PARTICIPANTS

Eight non-smoking male volunteers of normal BMI, aged 18 to 24 years, were recruited as per local ethical committee regulations (Ref 08/H0101/16) for experiment 1. They had no known medical conditions and were on no regular treatment, with no history of corticosteroid use.

Eight non-smoking male participants aged 18 to 25years in experiment 2 with the same exclusion criteria were also recruited to the OPTIMI study arm conducted in Zurich, Switzerland,with ethical permission granted by the University of Zurich.

MICRODIALYSIS PROCEDURE

A. **Subcutaneous microdialysis Equipment:** A portable CMA 107 microdialysis pump with a sterile CMA 106 pump syringe was connected to a sterile CMA 66 linear microdialysis catheter with polyarylethersulphone membrane of 30 millimetres length, 0.5 mm diameter, and molecular cut-off of 20 KDa CMA (Microdialysis AB, Stockholm, Sweden). Its inlet and outlet tubes, made of polyurethane, were 400 mm and 100 mm long respectively, and had 0.5 mm diameter. Perfusion fluid T1 (Microdialysis AB, Stockholm, Sweden), specific for peripheral tissue use, was used to perfuse the SC catheter. Flow rates of 2 µl/min and 1 µl/min were used for the first and second sets of experiments, respectively. 300 µl polypropylene vials (Royem Scientific limited, Luton, Bedford UK) were used for microdialysate collection and storage.

B. **Portable Automated Collection Device Assembly:** The distal end of CMA 66 was connected to the collection system using a 15-20 cm section of fluorinated ethylene propylene (FEP – Linton Instrumentation) tubing, and commercially available tubing connectors. The two types of tubing connectors namely, pink adapter (connects FEP segment to device inlet) and MAB-8 connector (connects probe outlet to FEP segment) were immersed in absolute ethanol for at least ten minutes prior to connection, as per manufacturer's recommedation

(Royem Scientific limited, Luton, Bedford UK). The above tubing assembly was used to connect microdialysis catheter to a novel automated collection device[16].

ASSAYS

Serum total cortisol concentrations were measured by electrochemiluminescent immunoassay (Cobas® e601 immunoassay analyser, Roche Diagnostics, Burgess Hill, UK). Intra-assay precision for serum concentrations of 208, 561 and 1268 nmol/l had CV of 1.3, 1.3 and 1.1 % respectively. The inter-assay precision for the same serum cortisol concentrations had CV of 1.6, 1.5, and 1.6 % respectively. Dialysate samples, decanted within 24 hours of collection and stored at -80°C, were analysed in singlicate samples using IBL ELISA kits for salivary cortisol (IBL, Hamburg, Germany), which was optimised for use of small dialysate volumes. The intra-assay precision for saliva concentrations of 7.45 and 64.58 nmol/l had coefficients of variation (CV) of 7.3 % and 3.1 % respectively. The inter-assay precision for saliva concentrations of 14.904 and 64.86 nmol/l had CV of 8.8 % and 6.4 % respectively.

EXPERIMENTAL DESIGN

STUDY PROTOCOL 1 (Fig. 1.): *Simultaneous determination of the rhythms of total plasma cortisol and subcutaneous free cortisol*. Participants arrived at the clinical research unit at least an hour in advance, and all cannulation was completed at least 45 minutes before beginning the experiment. The participants remained seated in a chair or reclining on a bed throughout the duration of sampling apart from during comfort breaks, when microdialysis sampling was not interrupted. Breakfast was served at 0700 (milk, cornflakes, banana/apple), lunch at midday (sandwich, orange juice, banana/apple) and hot ready meals at 1800 hours (Fig 2.). Lights were switched off between 23:00 and 07:00 hours. Subjects were allowed to carry out work-related activities on their personal computers when awake. An intravenous cannula for blood sampling was inserted in the left ante-cubital fossa. A microdialysis catheter was inserted subcutaneously in the lower anterior abdomen, and set up as described elsewhere[16]. Both serum and subcutaneous (SC) microdialysate samples were collected at ten-minute intervals in experiment one. The dialysate sampling clock-period was 18:55 on day 1 to 19:05 on day

2 (n=8). As blood sampling is episodic but microdialysate sampling continuous, the sample reading of the latter was considered to represent the midpoint of a given sampling duration e.g. sample timing of 10:05 was for dialysate obtained between 10:00 and 10:10.

STUDY PROTOCOL 2 (Fig. 3.): *Subcutaneous free cortisol profiles over 3 successive 24-hour periods in subjects at home*. A microdialysis catheter was inserted subcutaneously in the lower anterior abdomen, and set up as described elsewhere[16]. Sampling frequency of dialysate was every twenty minutes and duration was 72 hours, and no blood samples were collected. They attended the medical facility at the beginning for setup, then 24 and 48 hours later for replacing collection device battery and perfusion fluid and 72 hours after the start of the study time the apparatus was disconnected. Participants were allowed to carry out all activities as scheduled by them, including sleeping or meal times during the three days with restrictions only related to contact sport, running and swimming which were fortuitously not planned by any of the participants. Participants did not interrupt their normal daily routine and were free to choose their own meal times and meal composition. They kept a sleep diary and voluntarily reported unusual activities such as examinations, consumption of alcohol, and indulgence in leisure activities (poker, sexual activity).

STATISTICS

EXPERIMENT 1 (n=8): The only assumption made was that the direction of correlation was from serum to SC tissue i.e. the pattern in the former would precede the latter.

Statistical analyses were performed using the command-based program R[17]. Log-transformation of serum and SC values at each time with a point-wise mean was calculated. The SC time series was thought to have more 'noise' (short bursts of single readings not corresponding to the activity in serum) with significantly more unstable baseline, than the smoother profile of serum values. Kernel smoothing, for which time window of half an hour on each side of each log-transformed value was created and point-wise mean calculated, to minimise temporal noise. To examine the correlation between the two corresponding measurements, the data is assumed to be stationary (i.e. constant mean and variance). To induce stationarity the mean and variance were

158 stabilised by a differencing approach (i.e. between previous time's observation and the current time
159 observation). The cross autocorrelation function was used to compare the correlation of both time
160 series for varying time differences apart, known as lag. Each lag represented a difference of 10
161 minutes between the time points. Sine-wave fitting was used for detecting circadian rhythm and in
162 order to quantify how well the sine wave fits the data, the variance of the residuals for the sine model
163 was calculated (using the formula: 1-Var [sine model residuals]/Var[intercept model residuals]).
164 Another model for the relationship between log-transformed serum and SC free cortisol, a repeated
165 measures, mixed effects, generalised linear model was also used, as sine wave fitting is less flexible in
166 comparison.
167 EXPERIMENT 2 (n=8): Mean peak and trough values, by averaging the peak and trough values
168 in the daily profiles for each individual and the average difference between the peaks and
169 troughs were calculated. One-way ANOVA of peak, trough and the difference between the two was
170 carried out.
171

RESULTS

EXPERIMENT 1: On visual inspection of the profiles, the characteristic circadian rhythm is evident in both compartments, serum and SC with the lowest levels being achieved and maintained in the hours surrounding sleep onset. Sustained rise from such low levels leading to peak levels begins prior to lights being switched on, with peak levels occurring in every individual around waking – for most within an hour of waking (Figure 4. V41) but later, up to an hour after lunch, in the remaining few (Figure 4. V35). All participants showed a pulse, some similar amplitude and others lower than that around waking, at the time of midday meal (Figure 4).

A plot of the point-wise mean of the log-transformed serum and SC levels is shown in Figure 5. Both serum and SC cortisol levels display the typical profile with nadir around midnight following which increasing levels mount to the highest levels of the 24-hour period close to awakening time.

Using sine-wave fitting, circadian rhythm was evident in both body compartments i.e. serum and SC tissue in each individual, a typical example of which is depicted in Figure 6a and 6c, respectively. The pattern for the two compartments is the same but the amplitude of SC is lower as evidenced by the value of A in the sine wave function equation for the group [-0.87 for serum (Figure 6a) and -0.95 for SC (Figure 6d)] and for a typical individual [-0.87 for serum (Figure 6c) and -1.08 for SC (Figure 6c)].

The variance of the residuals for the sine model (Table 1) indicates that although the sine wave model is not ideal for either time series, it is a better fit (maximum values closer to 1) for log of serum values than for SC, a fact also reflected by goodness of fit values (sine serum model=0.603 and sine SC model=0.440).

While ascertaining the most important components of the model, time was undoubtedly an important feature, which, due to the circadian rhythm was a cubic orthogonal polynomial, of which linear (17.6, 95% CI: 12.5, 22.8) and cubic (-12.1, 95% CI: -14.9,-9.4) components of the polynomial time best describes the relationship (Table 2). The fixed intercept (5.1, 95% CI: 4.9, 5.3) represents the mean log serum level at the start of the study, if log SC free was 0.

There was a strong cross-correlation between the two time series in every individual (Figure 7), and this appeared to be highest at lag 5. As each process is an autoregressive process, there is a strong correlation between the observation at a particular time point and those preceding and following it.

EXPERIMENT 2: Figure 8 shows the mean free cortisol values for all 8 participants over the duration of the trial. It is important to reiterate the fact that these participants had their SC free cortisol samples collected whilst they were free to carry out their scheduled/spontaneous day-to-day activities with the only limitation of avoiding contact sport or water-based activities. It is evident from this figure that the circadian rhythm is present on each of the three days. This circadian rhythm is characterized by a nocturnal nadir in early hours around sleep onset, upward trend during the later part of sleep with the peak occurring at or soon after waking. According to the self-reported diaries, sleep period was divided into three: first to include the beginning of sleep for any individual (22:00-03:00); second when all individuals were asleep (03:00-07:00) and third when some were still asleep (07:00-11:00). The participants, as a group, were asleep for the longest duration on the third night. The mean peak level is achieved at a similar time i.e. 10:00-11:00, regardless of different waking times on the three mornings.

We compared the within subject variation in cortisol over the three days of this study. The mean values for the three days are superimposed in Figure 9.

Mean peak and trough values, and the average difference between the peaks and troughs are depicted in Figure 10. One-way ANOVA of peak, trough and the difference between the two showed no significant differences between the daily profiles (peak: $p=0.2887$ $F=1.380$; trough: $p=0.9907$ $F=0.009400$, difference: $p=0.2840$, $F=1.401$).

Each individual's profiles for the three 24-hour periods were superimposed to examine the day-day-variability. Only one participant of eight had recorded identical bedtime and waking-up times for the three nights (Figure 11, Participant identifier Z08). Only one individual (Figure 11, participant Z01) recorded the same time of waking on two out of three days but he could not continue sampling on day three and hence his data is incomplete.

Four out of eight individuals went to bed at the same time on all three study nights. Six out of eight individuals woke up at different times every day, ranging from one to four hours later/earlier than the previous morning. Participant Z04 (Figure 11) got up progressively later having spent longer in bed over the three days. His peak levels and times did not show much variation, but there was only a difference of ninety minutes between the recorded waking-up times on days one and three. Participant Z09 (Figure 11) got up earlier each day (150 minutes earlier on day three compared to day one) with shorter time spent in bed. His peak levels were achieved approximately three hours prior to waking on day one, whereas although the peak occurred at similar times on days two and three when he was out of bed, but later compared to day one.

DISCUSSION

Although there has been much interest in the ambulatory measurement of cortisol all other technologies are still at the proof of concept stage[18], this is the first study to show the dynamics of 24-hour measurements of SC free cortisol in healthy individuals. The ability to measure hormone levels across the 24 hours-especially across the early hours of sleep-is very important as the sleep-wake/activity cycle is an important part of the body's circadian regulation[19]. In man, under normal circumstances, cortisol begins to rise during the latter half of sleep continuing into the early awake phase, sometimes until about noon, gradually declining thereafter to low levels during the hours surrounding sleep onset[13,20]. In nocturnal species e.g. rodents, the pituitary-adrenal rhythmicity is also coordinated to their sleep-activity cycle[21], and this includes the free corticosterone fraction in the brain, subcutaneous tissue and intravenous compartments[14,15].

In our free-living ambulatory studies we have been able to demonstrate this characteristic circadian profile of cortisol in all subjects both in their total plasma cortisol and their subcutaneous free cortisol. The most active period of cortisol release into circulation was from about 05:00 to 14:00. From 14:00 HPA activity was variable between individuals - some showed consistent decline until 19:00 and others had further episodes of cortisol release. All participants also showed increased cortisol following the consumption of lunch. The lowest levels during all 24-hour periods were observed during the hours following lights switch-off, at the onset of sleep. There was no entrainment schedule prior to the study day in the first experiment, and on the study day during the awake period the subjects' activities included coursework, watching movies, listening to music and speaking to friends and family on the phone with standard meals being served. It is important to note that all of the participants reported undisturbed sleep through the night, with the collection device in the travel bag around their waist.

It is noteworthy that the circadian pattern of hormone levels is not smooth, but consists of pronounced secretory bursts[22]. This ultradian rhythm becomes measurable at sampling frequencies

that are more rapid than half of the hormone's half-life[23]. Our data reveal minimal ultradian activity of the axis around sleep onset[24] followed by a several-fold rise during late sleep-early waking period in all, as well as a post-meal surge at midday[25,26]. Apart from these three consistent secretory components, the remaining time-domains of the day are much more variable between individuals with some peaks late afternoon-early evening as have been previously reported in plasma[27,28].

This is also the first continuous study of free cortisol levels for 3 successive days. This was made possible by the use of a novel automated collection device, which is robust enough to allow ambulatory sample collection in individuals who are free to go about their day-to-day activities. Equally importantly, undisturbed sampling throughout the duration of sleep can now be achieved. This has the dual advantage of prolonged dynamic hormone measurements and also the ability to avoid the use of Clinical Research facilities which are unnatural environments that not only can affect levels of stress responsive glucocorticoid hormones both in rodents[14,15] and in human beings[29], but may also disrupt normal sleep patterns. Furthermore there are significant resource implications, both in terms of space availability and the cost incurred to use such specialist facilities.

There are of course other systems for ambulatory measurement of cortisol. The classic one is saliva-which has been very widely used, often 2 or 3 times per day, with or without the so-called cortisol awakening response (CAR). One such study reported higher CAR on a workday than on a weekend day i.e. on a day off work[30]. All of our sampling was done on weekdays with no intervening weekend day. Significant intra-individual variation in CAR across days has been reported elsewhere[31], but other studies measuring cortisol in saliva at multiple times during the day in individuals on several days have reported averaged values which prevents insight into the robustness of the rhythm across those days[32]. The fundamental difficulty in relying on CAR values, and in making meaningful conclusions from the results on multiple days is that 'a different value' may simply be a product of the timing of saliva collection at a different time point along an endogenous pulse. Susceptibility to disease based on calculation of the nature of a circadian profile from few timed samples, even when collected on multiple days, must be drawn with caution[33]. The limitations to the use of saliva cortisol in this particular context are that it cannot be collected during sleep (nadir phase of cortisol) and that it is impractical to collect them continuously over a prolonged period of

time. Other systems for ambulatory measurement of cortisol in a range of body compartments (saliva, plasma and sweat) have also been successfully tested[34], which in combination with rapid analytical techniques[18], especially those with electrochemical sensing, could potentially pave the way for continuous cortisol monitoring systems (akin to continuous glucose monitoring system). None of these has progressed beyond proof of principal at this time.

Our data is consistent with data from rodents in which SC free corticosterone on two consecutive days shows remarkable consistency of both circadian and ultradian rhythm[15]. A previous study over 3 days of cortisol rhythm (hourly measurements during sleep and 3 hourly when awake) has previously been measured in 31 medical students advised to maintain a regular sleep/wake pattern. This also showed remarkable consistency[35]. Since the relationship of sleep duration[34], quality of sleep[36]and circadian regulation is of considerable clinical importance, the regularity of HPA activity within each individual is of great interest.

The impact of variable daily routines on the day-to-day profiles of cortisol is not known. Our second study deliberately imposed no activity structure on participants other than to avoid contact sport during 72 hours of sampling period. They did not refrain from alcohol or other dietary ingredients, and were encouraged to lead as 'normal' a life as possible. A variety of activities including potentially stressful ones e.g. attending examinations, chairing a student body annual meeting, meeting with a tutor to discuss possibility of failing a term, chairing a meeting to allocate tasks towards organizing the high profile University annual ball, as well as leisure activities like playing poker, watching television/movies, and sexual activity were reported informally during the sampling period. It is striking how similar the day-to-day SC free cortisol profiles are despite such variations in their activities in the awake state as well as their variable duration and pattern of sleep.

Despite their different activities reported informally, there was a remarkable consistency in each individual's own profile, with the lowest levels later part of the evening and early sleep hours, and acrophase around awakening time. Two individuals (Figure 8 Z05 and Z06) appeared to have high levels of free hormone in the early part of the night, soon after their retrospectively recorded time of going to bed. For both these individuals, the levels of SC free cortisol on the remaining two nights

315 at the same time were significantly lower, in keeping with the expected levels for early hours of the
316 night.

317 Two methods, sine wave and generalised linear mixed model were used to evaluate circadian
318 rhythm in these participants, neither of which was entirely adequate for the purpose. Although the
319 sine wave was appropriate for comparing the serum and SC free cortisol profiles of an individual, it
320 was a better fit for serum values than for SC values. The generalised linear mixed model method was
321 employed to increase the generalizability of the model, but it proved less than satisfactory, and may
322 be improved by adding other parameters. Secretory peaks and hence ultradian activity was clearly
323 discernible visually and detected objectively in the serum compartment but not in the SC tissue
324 compartment, using two standard techniques namely, Pulsar and Deconvolution analyses. Both were
325 unsuccessful in detecting equal number of pulses in the SC tissue as in serum (data not shown). A
326 number of considerations are likely to influence this key finding as discussed below.

327 The nature of sampling with microdialysis is continuous, however a timed reading is an
328 aggregate of the dialysate collected over the period, in this case over 10 to 20 minutes. This may
329 potentially dampen a pulsatile component. The half-life of SC tissue free cortisol in man is not
330 known but the sharp pulses of free corticosterone seen in the brain of the rat[14] certainly suggests rapid
331 tissue elimination of this steroid. In man, the half life of serum total cortisol is 68[22] to 82.8 minutes[37]
332 and although binding to CBG may reduce the available fraction and therefore increase the elimination
333 of free cortisol, it is unlikely to be shorter than 10 minutes.

334 Within the normal range of CBG, the proportion of free hormone is determined by ambient
335 temperature which, at levels found in fever or local inflammation results in a proportional increase in
336 free fraction[3,38,39]. Both the rise in temperature[39] and the effect of neutrophil elastase released at sites
337 of inflammation[40] can result in increased local levels. The role of albumin assumes greater
338 significance when CBG is inactive either quantitatively or qualitatively[39,41], and is independent of
339 temperature but shows reduced affinity at acidotic pH[39].

340 A limitation of our study is that we excluded female participants. This was necessary in the
341 current study as natural fluctuations of oestrogen and progesterone related to the menstrual cycle
342 would have altered both the level of CBG and competed for binding to CBG respectively[42].

A potentially important factor in the regulation of tissue free cortisol is the 11β-HSD enzyme system. 11β-HSD-1 in adipose tissue can generate cortisol from inactive cortisone in vivo[43,44],while 11BHSD2 in the salivary glands increases the proportion of cortisone in saliva[45,46]. Dube *et al* showed that SC tissue of the abdomen has higher 11β HSD-2 activity than that of the leg in lean individuals[44]. 11β HSD-2 has been found in the human epidermis and can be induced on injury[47], but its role in modulating levels of active cortisol in interstitial fluid or the pulses of cortisol in skin is not known. Great care was taken during the insertion of the subcutaneous probe to avoid the adipose layers, although no direct visualisation techniques were employed to confirm catheter position. In future measurement of both cortisol and cortisone in the dialysates would be advantageous.

The nature of the SC tissue itself may have diffusion kinetics that do not readily transmit ultradian activity present in the serum. As cortisol is lipophilic it may exist to some extent as a 'depot' in the SC adipose tissue with 'slow release' of free hormone over time. In rodent studies[15], there is clear demonstration of simultaneous circadian and ultradian pulsatility of free corticosterone in both SC tissue and intravenous compartments. The only difference they found was slightly (15-20%) lower levels of SC tissue free hormone between 15:00 and 21:00hrs[15], when the levels are rising to the acrophase prior to their activity phase. The explanations for this may relate to increased clearance of free corticosterone as the authors suggest, or may also be a property of the rat SC tissue. The SC tissue of rat is highly vascular, unlike that of man and so synchronous rhythms in the former are not a surprise. In our validation of microdialysis methodology for SC free cortisol measurement, we have detectable free cortisol pulses in the intravenous compartment, which are likely to be transmitted to the SC tissue especially when the threshold for CBG binding in the plasma is exceeded.

The fact that pulsatility was more evident in serum than in the SC tissue could also relate to assay techniques. The RIA used for serum samples had superior sensitivity and specificity to the ELISA used for dialysates. Furthermore due to the small volumes obtained during our studies samples were analysed in singlicates, which would allow analytical errors, although through the optimisation procedure, there were no compromises on the quality and performance of each assay. However, due to the small size of the samples, and lower concentration of free cortisol in the small samples the signal

to noise ratio may not have been adequate to detect pulses in the dialysates. Hopefully this will be improved in the future by the use of ultrasensitive LCMS.

There are many reasons why it will be valuable to measure 24-hour cortisol rhythms in ambulatory subjects – not only to understand normal physiology, but also to diagnose pathology and improve therapy. In terms of normal physiology we need to understand normal changes associated with ageing[48,49], aspects of jet-lag and synchronisation to new time zones[48], and the need to understand what is normal for optimal use in patients. Furthermore, the use of microdialysis allows us to measure free 'active' cortisol in the compartment in which it has access to its receptors-so we can have a much clearer view of local regulation of glucocorticoid responsive processes.

In terms of pathology, there are multiple opportunities for use of 24-hour monitoring to improve diagnosis or therapy. Within endocrinology, obvious indications would include the diagnosis of Cushing's syndrome by a 24-hour or simply an overnight-series of cortisol measurements. Indeed we could clarify whether circadian rhythmicity is lost in Cushing's disease[50–52]. For cortisol replacement therapy we could get a much clearer view of what is happening at tissue level in response to different replacements regimes[53,54]. Other indications would include investigations of adrenal incidentalomas and congenital adrenal hyperplasia. In addition to the endocrine indications we hope this technique may help in other conditions associated with disorders of the HPA axis such as depression[55], sleep disorders and recovery of the HPA axis following glucocorticoid therapy induced HPA suppression. We could also learn more about the physiological deviation from normal rhythm, usually temporary, seen in individuals recovering from major surgery[56] including cardiac surgery[57] and diseases of the cardiovascular system[58].

This is the first evidence of continuous measurement of free cortisol for 3 consecutive days in healthy people outside of a research facility setting, free to carry out their routine activities without major limitations. We have been able to demonstrate that free cortisol in the SC tissue shows remarkable consistency despite varied daily routines and activities of individuals. This provides considerable scope to plan future studies to investigate disease and therapeutic responses of the HPA axis secure in the knowledge that there is relatively little intra-individual variation even in individuals whose sampling days contain dissimilar activities/routines.

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TABLES AND FIGURES

Figure 1. PROCEDURES FOR EXPERIMENT 1. Intravenous cannula was inserted in the left antecubital fossa. Microdialysis catheter was inserted subcutaneously in the lower anterior abdominal wall and connected to microdialysis pump and collection device as described previously. All procedures were complete and set up at least 45 minutes prior to commencing the experiment.

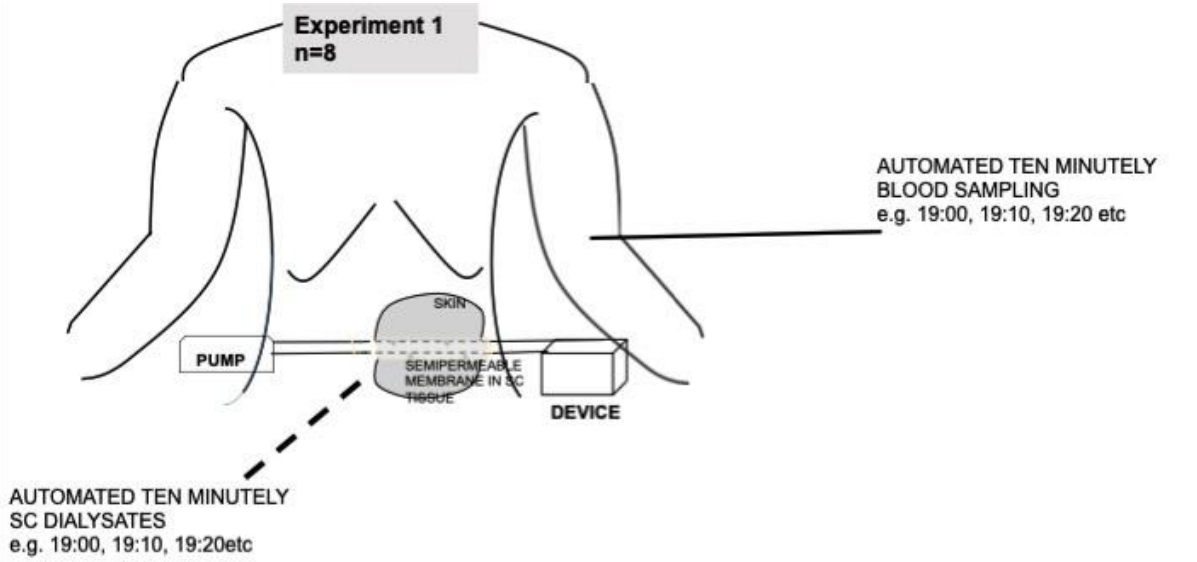

Figure 2. SCHEMATIC FOR EXPERIMENT 1. Sampling commenced at 18:55 for microdialysate and at 19:00 for serum and completed at 19:05 and 19:00 respectively. Meals were served at the time denoted along the X axis. Lights were switched off at 23:00 and back on at 07:00.

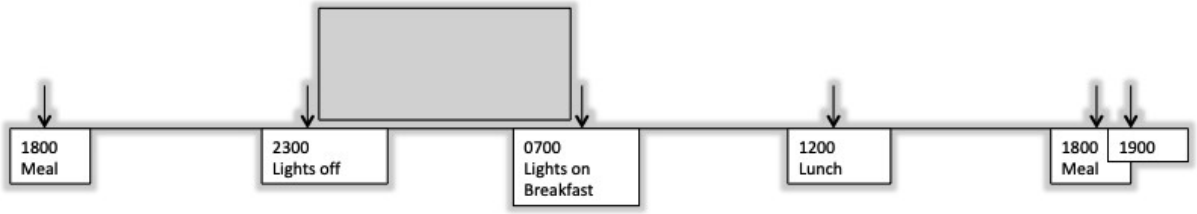

Figure 3. SCHEMATIC FOR EXPERIMENT 2.Microdialysis system was set up by 17:00 with a sampling duration of 72 hours from 18:00 on day 1 to 18:00 on day 3 when the experiment terminated. Each participant attended the clinical facility between 16:00 and 18:00 for change of cartridge. Time for retiring to bed and waking up were recorded separately in a sleep diary.

Figure 4. **CIRCADIAN PROFILE OF SERUM TOTAL AND SUBCUTANEOUS FREE CORTISOL IN TWO HEALTHY INDIVIDUALS**. Clock time is displayed along the X-axis and serum total cortisol on left and SC free cortisol (TISSUE) on the right Y-axes. Shaded area represents the lights-off period. Meal times are represented by dotted lines at 0700, 1200 and 1800.

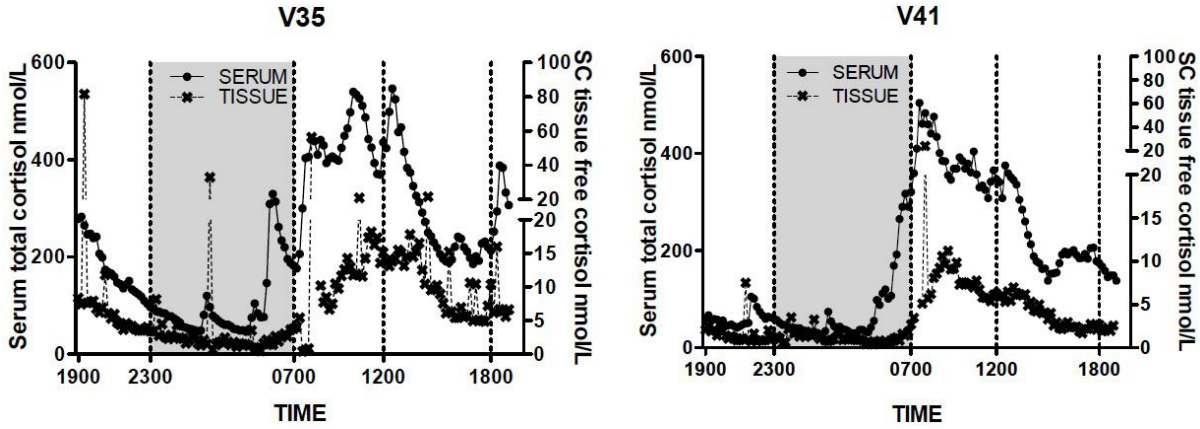

Figure 5. **PLOT OF LOG TRANSFORMED SERUM TOTAL (LEFT plot) AND SC FREE (RIGHT plot) CORTISOL LEVELS**. Time is displayed along the X-axis. 2300 to 0700 denotes the lights off period. Dotted lines indicate meal times (breakfast at 07:00, sandwich lunch at 12:00 and hot meal at 18:00). Bold line is the Kernel smoothed estimate of the raw mean for each time point for 8 individuals and shaded area represents 95% confidence intervals.

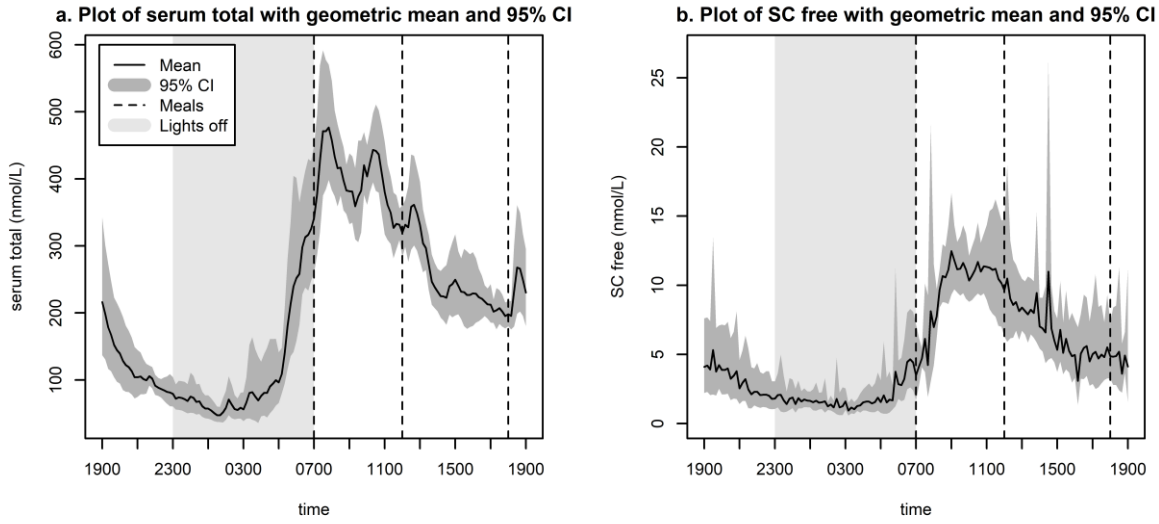

Figure 6. SINE WAVE FITTING: Fitted sine wave of log of serum total cortisol (a-single individual; c-group) and log of sc free cortisol (b-single individual; d-group). Solid line indicates mean log values and the dotted line indicates log values for each individual.

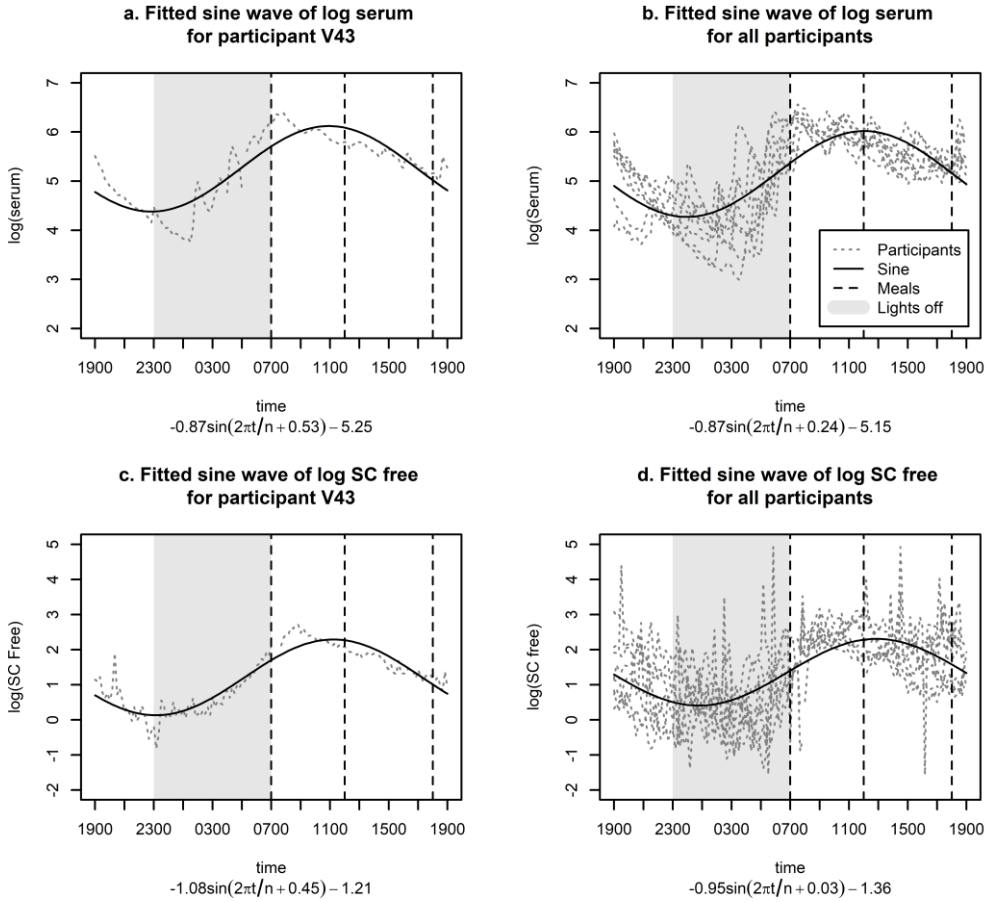

Table 1. RATIO BETWEEN THE VARIANCE OF THE RESIDUALS FOR THE SINE WAVE MODEL FOR SERUM AND SC TIME SERIES. Lower values indicate a better model fit to the data.

TABLE 1. TABLE OF THE RATIO BETWEEN THE VARIANCE OF THE RESIDUALS FOR THE SINE MODEL BY PARTICIPANT		
PARTICIPANT ID	SERUM	SC
V31	0.703	0.579
V35	0.712	0.563
V36	0.631	0.568
V38	0.733	0.349
V40	0.598	0.691
V41	0.803	0.598
V42	0.683	0.737
V43	0.786	0.859

Table 2. GENERALISED LINEAR MODEL OF RELATIONSHIP BETWEEN SERUM AND SC FREE CORTISOL. Model coefficients estimates, standard error, 95% confidence interval and p-value from the mixed effects repeated measures generalised linear model of the relationship between log SC free and log serum total with a random intercept for each participant.

TABLE 2. TABLE OF FIXED EFFECTS FOR THE GENERALISED LINEAR MODEL OF THE RELATIONSHIP BETWEEN SERUM AND SC FREE LEVELS OVER A 24-HOUR PERIOD					
COVARIATE	VALUE	STD. ERROR	LOWER CI	UPPER CI	p-VALUE
Intercept	5.121	0.103	4.919	5.323	<0.001
Log (SC Free) $_{t-5}$	0.020	0.008	0.004	0.036	0.015
Time (poly1)	17.638	2.631	12.48	22.795	<0.0001
Time (poly2)	1.805	1.836	-1.794	5.405	0.325
Time (poly3)	-12.133	1.406	-14.89	-9.377	<0.001

Figure 7. **CROSS AUTO-CORRELOGRAM OF SERUM AND SC FREE CORTISOL FOR EACH INDIVIDUAL PARTICIPANT** (n=8). The values of cacf are along the Y-axis and time lag along the X-axis. Time lag of +1 is an interval of 10 minutes. The values of each correlogram at all time points exceed the confidence interval (dotted line) suggesting a strong correlation between the two profiles in every individual.

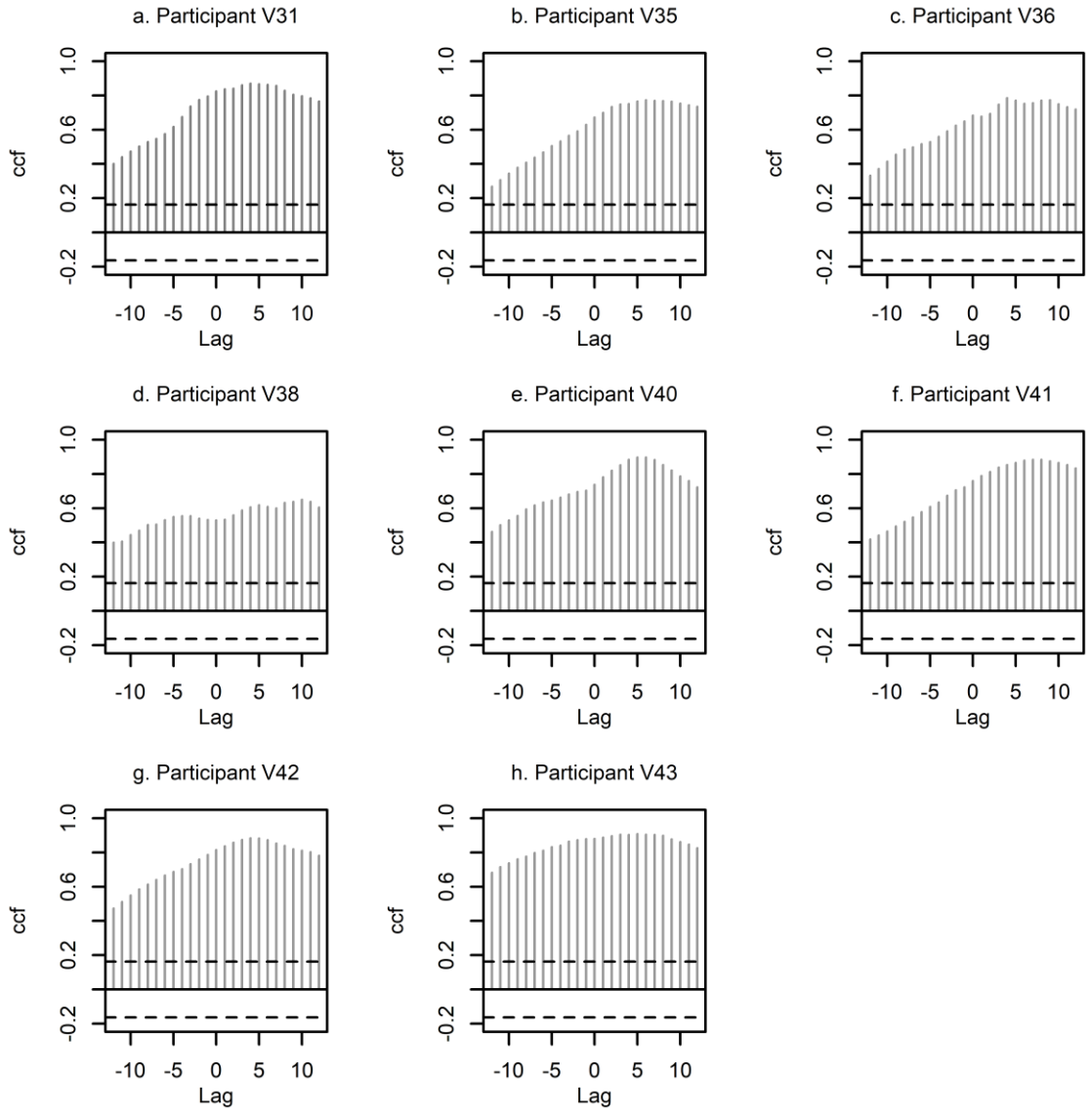

Figure 8. **MEAN SC FREE CORTISOL VALUES FOR ALL PARTICIPANTS (n=8) OVER THREE DAYS**. Light grey areas represent the time when some, but not all (dark grey), participants were asleep. Clock time is along the X-axis and SC free cortisol (nmol/L) along the Y axis.

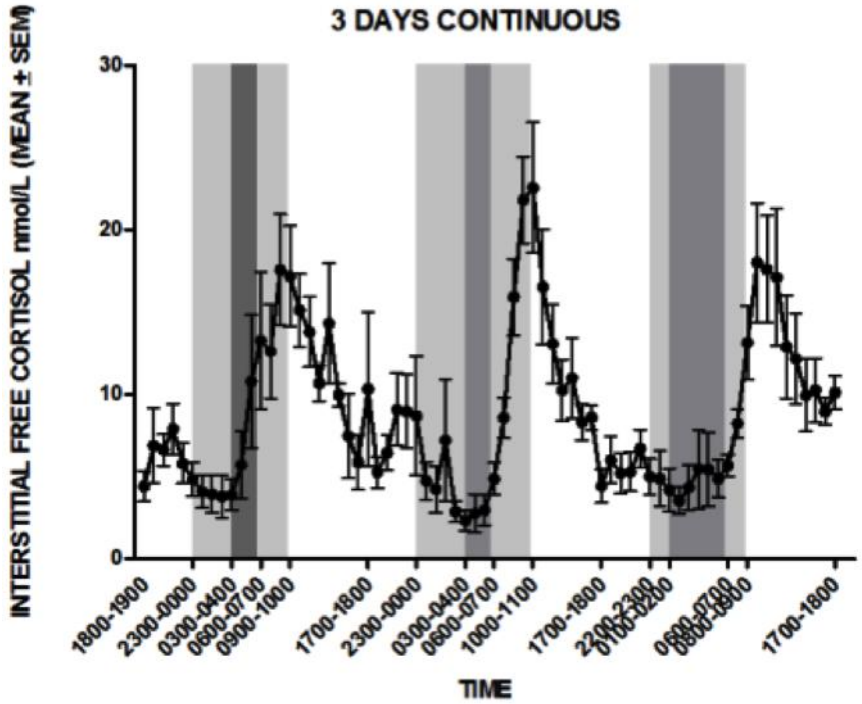

Figure 9. **MEAN ± SEM SC FREE CORTISOL VALUES FOR ALL PARTICIPANTS (n=8) OVER THREE DAYS SUPERIMPOSED**. Light grey areas represent the time when some, but not all (dark grey), participants were asleep. Clock time is along the X-axis and SC free cortisol (nmol/L) along the Y axis.

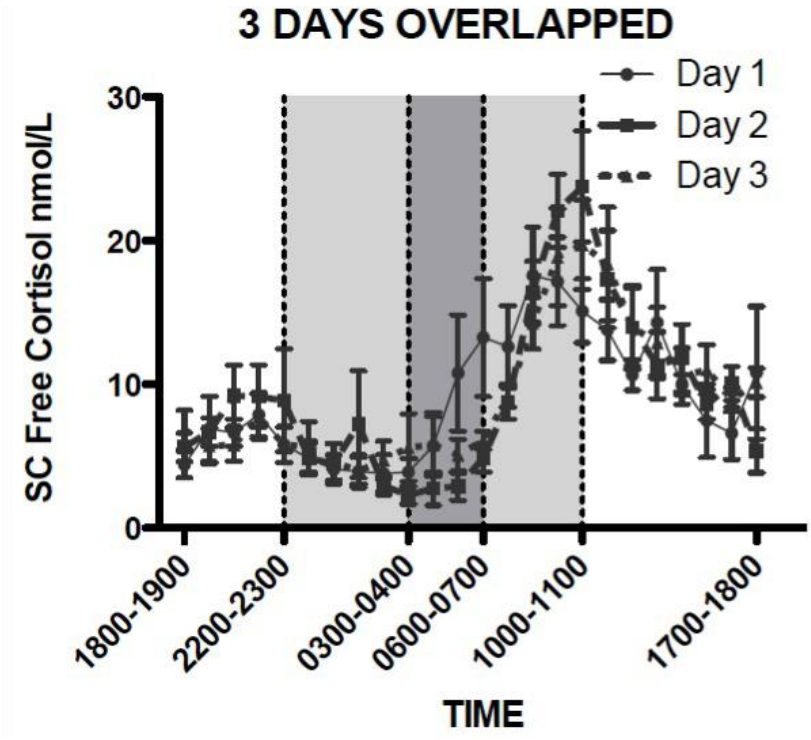

Figure 10. **AVERAGE PEAK, TROUGH AND PEAK MINUS TROUGH VALUES FOR 3 DAYS (n=8)**. SC free cortisol values are along the Y-axis and the parameters calculated are along the X-axis as labelled. Days are denoted as D1, D2, D3.

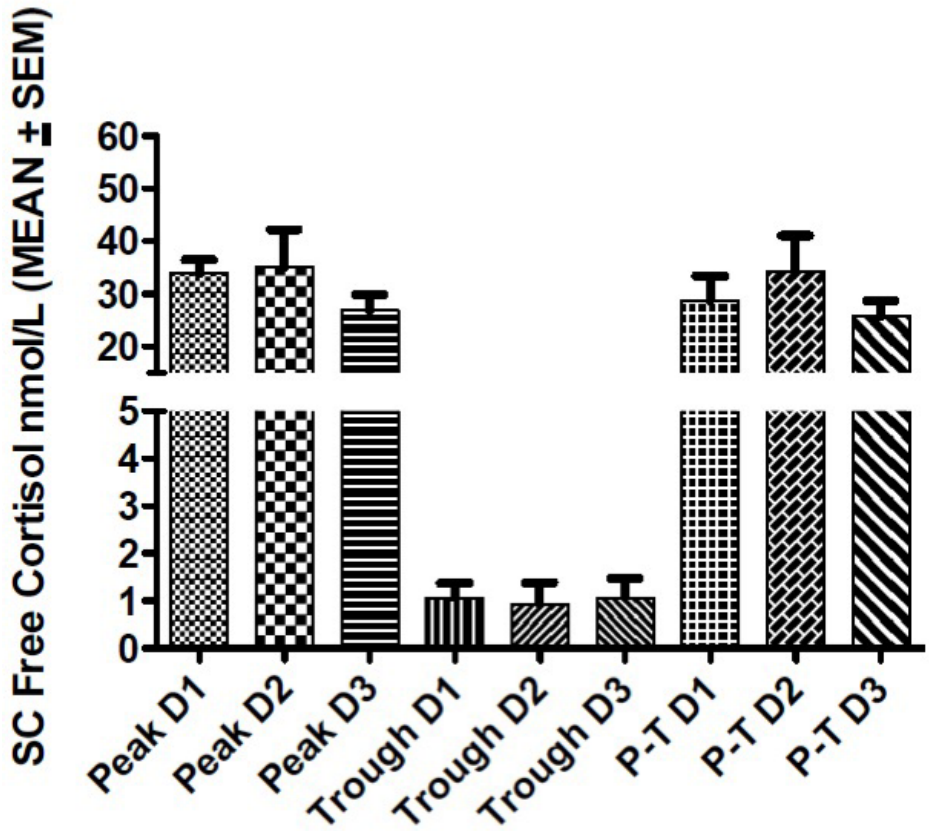

Figure 11. **INDIVIDUAL SC FREE CORTISOL VALUES FROM DAYS 1 TO 3 OVERLAPPED**. SC free cortisol is along the Y-axis and clock time along the X-axis. Shaded area represents the time an individual was asleep on all three nights. Verticals lines represent recorded bedtime and wake-up times, if outside of the shaded area.